Detection of *Anisakis simplex*-Induced Basophil Activation by Flow Cytometry

Miguel Gonzalez-Muñoz,[1*] Rosario Luque,[2] Frans Nauwelaers,[3] and Ignacio Moneo[1]

[1]Department of Immunology, H. Carlos III, Madrid, Spain

[2]BD Biosciences Europe, Madrid, Spain

[3]BD Biosciences Europe, Erembodegem, Belgium

Background: Laboratory diagnosis of anisakidosis is based on specific serum IgE detection. Recently, detection of allergen-induced basophil activation by flow cytometry has been proposed as a valuable tool for allergy diagnosis.

Objective: To evaluate if detection of *Anisakis*-induced basophil activation by flow cytometry is a useful tool in the diagnosis of *Anisakis* allergy.

Methods: Patients with *Anisakis* allergy (A.s.$^+$, $n = 37$), patients reporting chronic urticaria or abdominal pain unrelated to fish ingestion (A.s.$^-$, $n = 51$), and healthy controls ($n = 12$) were studied. Specific IgE to *Anisakis simplex* (*A. simplex*) was quantified with CAP-FEIA method, and basophil activation test was performed with three different concentrations of an *Anisakis* crude extract. Basophil gating was performed with CD123 and HLA-DR, and cellular activation was measured with CD63.

Results: A.s.$^+$ patients showed significantly higher age and total IgE levels than did the A.s.$^-$ patients. Specific IgE to *A. simplex* correlated with the activated basophil percentages obtained with 15 μg/mL ($r = 0.80$; $P < 0.001$), 1.5 μg/mL ($r = 0.84$; $P < 0.001$), and 0.15 μg/mL ($r = 0.82$; $P < 0.001$) of *A. simplex* crude extract. Nine individuals (3 in the A.s.$^+$ group and 6 in the A.s.$^-$ group) were nonresponders to basophil stimulation with anti-IgE. Five A.s.$^-$ patients showed positive IgE values to *A. simplex* while the basophil activation test was negative.

According to the receiver operating characteristics curves performed between A.s.$^+$ vs. A.s.$^-$ and A.s.$^+$ vs. healthy controls, the cutoff for a positive basophil activation test was $\geq$21% (specificity = 96%, sensitivity = 100%), and 16% (sensitivity and specificity of 100%) respectively. When nonresponders were included in the A.s.$^+$ vs. A.s.$^-$ analysis, sensitivity decreased to 95%. Multivariate logistic analysis showed that the specific basophil activation was a factor independently associated with clinical symptoms of *A. simplex* allergy.

Conclusions: Detection of *A. simplex*-induced basophil activation by flow cytometry is a useful laboratory technique for the diagnosis of anisakidosis, supplementing specific IgE determinations. © 2005 International Society for Analytical Cytology

Key terms: *Anisakis simplex* allergy; CD63; basophil activation; Immunoglobulin E

Anisakis simplex (*A. simplex*) is a fish parasite responsible for human infection (anisakidosis) and is able to induce IgE-mediated reactions with several clinical manifestations ranging from urticaria or angioedema to anaphylaxis (1–3). The involvement of an IgE-mediated response to *A. simplex* has been proven by skin prick test, serum specific IgE detection, and histamine release in whole blood (1–9). The two most frequently used methods in *Anisakis* allergy diagnosis are the skin prick test and the detection of specific IgE using whole antigen from the parasite. Unfortunately, these diagnostic tools are interfered with cross-reactivities with other nematodes and micro organisms (10–15).

Over the last years, the identification of allergen-induced basophil activation by flow cytometry has been assessed as a new approach to the allergy diagnosis (16).

The method is based on the detection of a changed phenotype on activated basophils. When basophils are incubated with allergens, the specific allergen is recognized by surface receptor FcεRI-bound IgE. As a consequence, the activated basophil secretes bioactive mediators and up-regulates membrane expression of certain markers. The most commonly used marker in basophil activation tests is CD63, a granule protein that is expressed on the basophil plasmatic

*Correspondence to: M. Gonzalez-Muñoz, Department of Immunology, H. Carlos III, Sinesio Delgado 10, 28029-Madrid, Spain.

E-mail: mgonzalez_munoz@hotmail.com

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membrane following activation, and its expression has been shown to correlate with histamine release (17,18).

The aim of this study is to assess the usefulness of flow cytometry to analyze in vitro allergen induced activation of basophils in *A. simplex* allergy. Receiver operating characteristics (ROC) curve analysis was performed, and factors independently associated with clinical history of *Anisakis*-induced allergy were identified.

MATERIALS AND METHODS

Subjects

Eighty-eight consecutive patients (28 male/60 female; median age 34, IQR = 28–48) referred to the department of immunology to assess if *A. simplex* was involved as a causing agent of their symptoms (urticaria being the most frequently reported) were included in the study. *A. simplex* allergy was considered when the following criteria were met: compatible clinical history, *A. simplex* positive specific IgE values, and negative for specific IgE against fish. Thirty-seven patients had a clinical history of *A. simplex* allergy (A.s.$^+$), reporting several episodes of severe abdominal pain, vomiting and/or acute urticaria-angioedema within 12 h of eating undercooked or raw fish. *A. simplex* allergy was confirmed by detecting specific IgE, and these patients were considered as sensitized to the parasite. The remaining 51 patients reported chronic urticaria or abdominal pain unrelated to fish ingestion (A.s.$^-$). *A. simplex*-specific IgE was found in five of these patients. Twenty-four of the total 88 patients reported symptoms suggestive of hypersensitivity to other allergens, that were confirmed by detecting serum specific IgE (grass pollen, $n = 10$; grass pollen and mite, $n = 1$; mite, $n = 3$; peanut, $n = 3$; wheat, $n = 1$; shrimp, $n = 5$; shrimp and grass pollen, $n = 1$). Fish allergy was excluded, analyzing specific IgE in all these patients. Twelve healthy controls with no previous history of allergy and negative for specific IgE to *A. simplex* were included in the study. Basophil activation with *A. simplex* crude extract was assessed in all patients and controls by flow cytometry.

Preparation of Anisakis Crude Extract

Larvae were manually extracted from the muscle tissue of *Merluccius merluccius* obtained from local markets, as previously described (19). One gram of larvae was mixed with 10 mL of PBS and ground in a mortar. The mixture was centrifuged for 5 min at 1,000*g*, and the pellet was discarded. The protein content of the supernatant was 2 mg/mL, as measured by the method of Bradford (20) with BSA as a standard. The crude extract of the parasite was characterized by immunoblotting as previously described (19). Pooled sera ($n = 3$) of A.s.$^+$ patients indicated the presence of several parasite allergens, and no protein was recognized in the pooled sera of healthy controls.

Quantification of Total and Specific IgE

Serum specific IgE determinations were performed by UniCAP (Pharmacia-Upjohn, Uppsala, Sweden) according to the manufacturer's instructions. The detection range was 0.35–100 kUA/L. Values greater than 0.35 kUA/L were considered positive. Total IgE was quantified with an enzyme immunoassay (Access Total IgE, Beckman Coulter, USA). The detection range was 0.25–3,000 IU/mL.

Basophil Activation Test

Heparinized blood was drawn and basophil activation test was performed within 3 h of venipuncture. 100 μL of sample was incubated for 15 min at 37°C with 20 μL stimulation buffer containing IL-3 (60 ng/mL, Pharmingen/BD Biosciences, Erembodegem, Belgium) and *A. simplex* crude extract (15, 1.5, 0.15 μg/mL final concentrations). Negative and positive controls were included by incubating the blood without allergen and 10 μL anti-IgE (10 μg/mL, Pharmingen), respectively. Basophil activation was stopped by adding 10 μL of EDTA (20 mM; Sigma-Aldrich, Madrid, Spain) at room temperature for 5 min.

To analyze basophil activation, cells were stained with 20 μL CD63(IgG1)-FITC/CD123(IgG1)-PE/HLA-DR(IgG2a)-PerCP reagent mixture (BD Biosciences) for 20 min in the dark on ice. Then, samples were incubated with 2 mL FACS Lysing solution (BD Biosciences) for 15 min at room temperature. After centrifugation (300 g, 5 min) and washing with PBS, cells were resuspended with 300 μL of 1% paraformaldehyde (Sigma) and acquired in a FACScan flow cytometer (BD Biosciences).

To analyze the percentage of allergen-activated cells, the basophil population was gated using side scattering (SSC) and CD123 to eliminate most of the debris and select CD123$^+$ cells. At least 500 CD123$^+$ cells were analyzed. Then, cells gated were displayed in a CD123/HLA-DR dot plot to eliminate HLA-DR$^+$ cells. Subsequently, CD63 expression was detected in the CD123$^+$ HLA-DR$^-$ basophils. In the CD63/CD123 dot plots the quadrants were set with the negative control of the test so as to identify the activated basophils by high CD63 expression (3rd–4th decades; Fig. 1). Percentage of activated basophils was calculated by subtracting spontaneous CD63 expression (negative control) from the data obtained with *Anisakis* stimulation.

Endotoxin is known to enhance IgE-mediated basophil degranulation (21). To exclude potential contamination of the parasite crude extract by endotoxin, basophil activation test was performed by incubating blood samples (8 healthy controls) with 10 μL suboptimal concentration of anti-IgE (1 μg/mL) with or without 15 μg/mL of parasite crude extract.

Statistical Analysis

Statistical analysis was performed with SPSS 10.0 software (Chicago, IL). Shapiro–Wilks test was performed to analyze normality of parameters. Median (range) de-

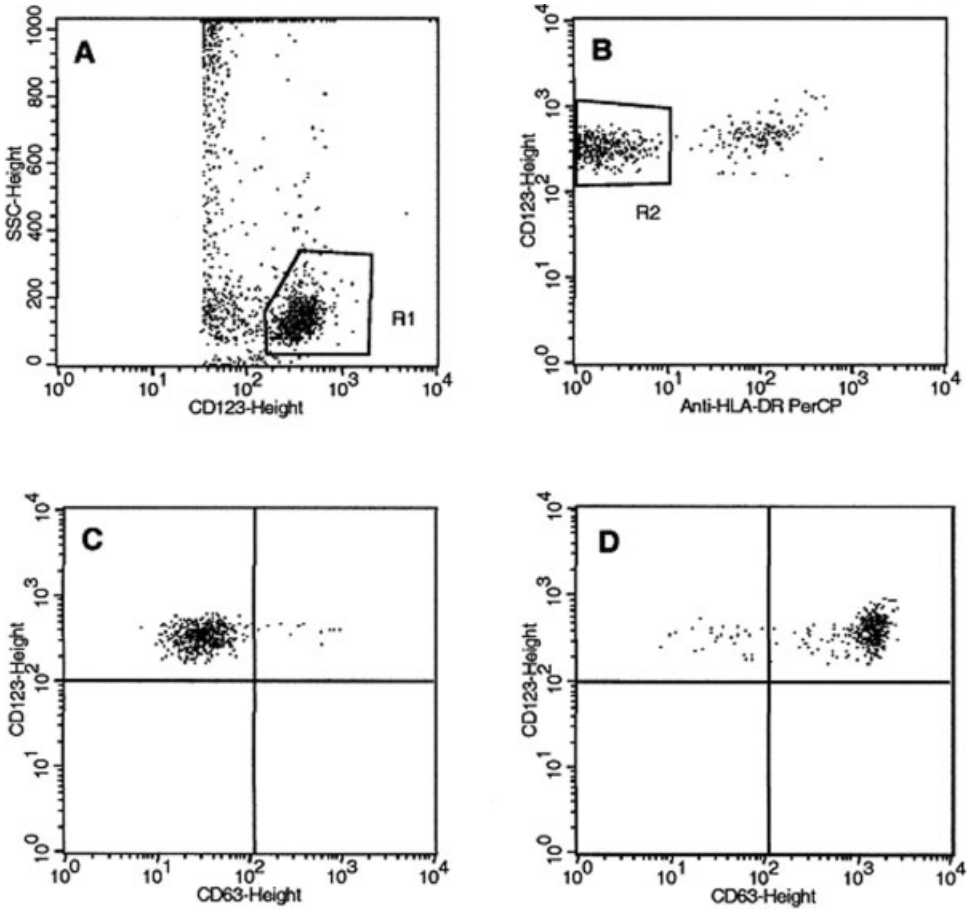

FIG. 1. Analysis of basophil activation by flow cytometry. **A**,**B** show gating strategy for basophil identification. First, basophils are gated as a cell population with low SSC and high CD123 expression (A, R1). Then, basophils are selected as HLA-DR negative cells (B, R2). **C**: a representative case of A.s.$^-$ patients. **D**: a representative case of the A.s.$^+$ patients.

scribed continuous variables. Qualitative variables were compared with χ^2 test. Intergroup comparisons of continuous variables were performed with the Mann-Whitney *U* test and bivariate correlation was analyzed with the Spearman's rank test. Receiver operating characteristics (ROC) curve analysis was performed to calculate the optimal cutoff value of activated basophils, corresponding to the greatest sum for specificity and sensitivity values. Univariate analysis was performed to evaluate risk factors associated with *Anisakis* allergy. Variables tested were sex, age >35 years, total IgE >200 IU/mL, detection of specific IgE to *A. simplex* and detection of specific IgE to other allergens, and basophil activation with *Anisakis* crude extract.

A stepwise multivariate logistic analysis (forward: Wald) was performed to evaluate independent predictors for *Anisakis* allergy. Explanatory variables were: sex, age, specific IgE to *A. simplex*, specific IgE to other allergens, and basophil activation with *Anisakis* crude extract. Variables with no significance at the 0.1 level were removed from the final model.

RESULTS

Flow cytometry technique has shown to be a reliable tool in the detection of basophil activation using CD63/CD123/HLA-DR. Basophils were identified as low side scattering (SSC), CD123$^+$, and HLA-DR$^-$ cells. Gating strategy and positive and negative representative results are shown in Figure 1.

To analyze whether the parasite crude extract was potentially contaminated by endotoxin, we compared basophil activation induced by anti-IgE alone with that induced by both anti-IgE and the parasite crude extract. No significant differences ($P = 0.95$) in the percentage of activated basophils were found between anti-IgE (32%, 10–86%) and anti-IgE plus the crude extract (30%, 10–81%) stimulation. Thus, we conclude that there is no synergistic effect of the crude extract on basophil activation because of the presence of endotoxin.

Table 1 shows the comparison of demographic and laboratory data between A.s.$^+$ and A.s.$^-$ patients. The A.s.$^+$ group showed significantly higher age and total IgE levels than did the A.s.$^-$ group. No differences were found in sex and frequency of positive specific IgE to allergens other than *A. simplex* between both groups. Positive and negative controls in the basophil activation test showed no differences between both groups. Nine individuals (3 in the A.s.$^+$ group and 6 in the A.s.$^-$ group) were nonresponders to basophil stimulation with anti-IgE. Of note, two subjects showed high percentage of activated basophils (49 and 82%) in the negative control. Total IgE correlated with specific IgE to

Table 1
Demographic and Laboratory Data of the Patients

	A.s.$^-$ patients ($n = 51$)	A.s.$^+$ patients ($n = 37$)	P
Sex (m/f)	14/37	14/23	0.350
Age (years)	32 (2–86)	40 (18–70)	0.005
Total IgE (IU/mL)	94 (1 to >3000)	308 (9 to >3000)	0.018
Specific IgE to *Anisakis* (kUA/L)	<0.35 (<0.35–81.5)	11.2 (0.9 to >100)	<0.001
Specific IgE to other allergens	12 (24%)	12 (32%)	0.340
Activated Basophils (%)[a]			
Basal	2 (0–82)	2 (0–49)	0.936
Anti-IgE	75 (16–99)	85 (18–94)	0.789
15 μg/ml *Anisakis*	6 (0–91)	90 (22–97)	<0.001
1.5 μg/ml *Anisakis*	1 (0–30)	89 (23–96)	<0.001
0.15 μg/ml *Anisakis*	0 (0–17)	50 (2–96)	<0.001

[a]Nine nonresponder patients (3 in A.s.$^+$ group and 6 in A.s.$^-$ group) were not included in the description of activated basophil values.

A. simplex ($r = 0.54$; $P < 0.001$) and percentage of activated basophils with 15 μg/mL ($r = 0.30$; $P = 0.021$), 1.5 μg/mL ($r = 0.37$; $P = 0.004$) and 0.15 μg/mL ($r = 0.28$; $P = 0.040$) of crude extract of the fish parasite. Specific IgE to *A. simplex* closely correlated with the percentage of activated basophils with 15 μg/mL ($r = 0.80$; $P < 0.001$), 1.5 μg/mL ($r = 0.84$; $P < 0.001$), 0.15 μg/mL ($r = 0.82$; $P < 0.001$) of *A. simplex* crude extract. Five A.s.$^-$ patients had specific IgE to *A. simplex* and negative basophil activation. Two of them showed low values of *Anisakis* specific IgE (0.46 and 0.62 kUA/L), whereas the remaining three had high *Anisakis* specific IgE levels (2.42, 3.91, and 81.5 kUA/L). These three patients were parasitized by *A. lumbricoides* (as determined by parasitologic exam) and had elevated levels of *A. lumbricoides*-specific IgE (4.88, 16.8, and >100 kUA/L).

ROC curve analysis was performed between A.s.$^+$ and A.s.$^-$ groups to calculate the optimal threshold value of activated basophils with the addition of *A. simplex* crude extract.

The areas under the curve for 15, 1.5, and 0.15 μg/mL of crude extract were 0.96 (95% confidence interval [CI]: 0.92–0.99; $P < 0.001$), 0.99 (95% CI: 0.99–1.00; $P < 0.001$) and 0.97 (95% CI: 0.94–1.00; $P < 0.001$), respectively. Threshold values of activated basophils providing the maximal specificity and sensitivity were the following: 33% for 15 μg/mL (sensitivity = 97%, specificity = 78%), 21% for 1.5 μg/mL (sensitivity = 100%, specificity = 96%), and 8% for 0.15 μg/mL (sensitivity = 91%, specificity = 89%). When nonresponders were included in the analysis, the following sensitivity and specificity values were obtained: 92 and 80% for 15 μg/mL, 95 and 96% for 1.5 μg/mL, and 86 and 90% for 0.15 μg/mL. According to this data, the best percentage of activated basophils discriminating A.s.$^+$ and A.s.$^-$ patients was 21% when 1.5 μg/mL crude extract is used (Fig. 2). ROC analysis between A.s.$^+$ patients and healthy controls yielded a cutoff of 16% activated basophils (1.5 μg/mL crude extract) with a sensitivity and specificity of 100%. The sensitivity was reduced to 97% when nonresponders were included in the analysis.

Univariate analysis was performed to analyze factors associated with *A. simplex* allergy. Variables were sex, age >35 years, total IgE >200 IU/mL, specific IgE to *A. simplex* ≥0.35 kUA/L, positive specific IgE to other allergens, and positive basophil activation. Only specific IgE to *A. simplex* (relative risk [RR] 10.2, 95% CI: 4.4–23.4; $P < 0.001$) and basophil activation (RR 22.5, 95% CI: 5.8–87.2; $P < 0.001$) were found to be associated with clinical anisakidosis.

Multivariate analysis to search for factors independently associated with *A. simplex* allergy were performed using as covariates sex, age, total IgE values, detection of specific IgE to allergens other than *A. simplex*, *Anisakis*-specific IgE levels, and activated basophil values. Only basophil activation was shown to be independently associated (adjusted OR 1.3, 95% CI: 1.02–1.66; $P = 0.031$) with *Anisakis* allergy.

DISCUSSION

Flow cytometric analysis of allergen-induced basophil activation has been reported for allergies to pollen (22–25), latex (26,27), house dust mite (22,24), drugs (28–31) and venoms (17,32), and it has been proven helpful for diagnosis of allergy. This work shows that flow cytometric analysis of CD63 up-regulation in *A. simplex*-activated basophils is a useful approach for anisakidosis diagnosis. Monoclonal antibodies chosen for basophil identification by flow cytometry were CD123 (IL3 receptor α-chain) and HLA-DR. This approach is based on a previous report (33) describing a method for identifying dendritic cells. Among peripheral blood mononuclear cells with low SSC, high expression of CD123, and negative for HLA-DR, the authors observed (besides dendritic cells) a population that was identified as basophilic granulocytes. Later, this finding was applied to the analysis of basophil activation by flow cytometry (34–36).

We performed the study with samples from consecutive patients referred to our department to evaluate this assay in a regular daily practice. Correlation analysis showed a low association between total IgE and the percentage of activated basophils that could be accounted by the correlation observed between total and specific IgE. Furthermore, we found a positive correlation between specific IgE and the percentage of activated basophils and this association has also been reported in wasp venom allergy (37), although this relationship is

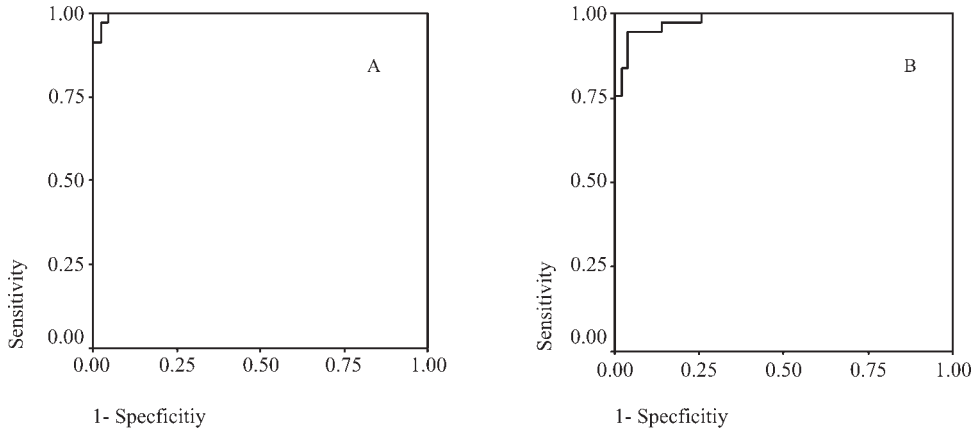

FIG. 2. ROC curve analysis for *A. simplex*-induced CD63 expression generated between A.s.$^+$ and A.s.$^-$ patients. **A**: an optimal threshold of 21% of activated basophils was generated, yielding a sensitivity of 100% and specificity of 96%. **B**: sensitivity decreased to 95% when nonresponder (no basophil activation with anti-IgE) subjects were included in the analysis.

not a general finding (27). A sensitivity of 100% and specificity of 96% were obtained when the cutoff is set at 21% of activated basophils according to the ROC curve analysis performed between A.s.$^+$ and A.s.$^-$ patients. The basophil activation test discriminated the A.s.$^+$ and healthy controls with 100% sensitivity and specificity. Three of the 37 allergic patients were nonresponders (no activation with anti-IgE). In these cases the test provided no information on their sensitization; however, for study purposes, they were considered as false negative results. Therefore, their inclusion reduced the sensitivity of the test to 95%. The high sensitivity and specificity found with *A. simplex*, similar to those reported with other allergens (23–27), support this technique as a useful tool for anisakidosis diagnosis.

Elevated *Anisakis*-specific IgE was found in three A.s.$^-$ who showed negative basophil activation with *A. simplex* crude extract. This could be explained by nonspecific adsorption due to high total serum IgE values or by cross-reactivity between *A. simplex* and other organisms (10–15). Both mechanisms can be involved in these false positive cases because their sera contained *A. lumbricoides*-specific IgE and two of them showed total serum IgE >3,000 IU/mL. Up to 50% of false positive CAP results have been reported when studying sera from *Anisakis*-free patients but having several parasite infections (15). Moreover, antigenic relationship between *A. simplex* and ascaridoids has been reported (10,38,39) and, therefore cross-reactivity can account for the *Anisakis*-specific IgE found in these three A.s.$^-$ patients.

Data supporting the usefulness of this technique in the evaluation of the clinical relevance of the serum specific IgE were obtained from the multivariate analysis. Basophil activation results were found to be independently associated with the presence of anisakidosis symptoms. This finding indicates that the basophil activation technique mimics the in vivo mechanisms that are at the cause of the clinical symptoms. On the other hand, the multivariate analysis did not identify any association between positive specific IgE and the presence of clinical symptoms. The usefulness of basophil activation test to assess the clinical relevance of positive IgE values has also been proposed for latex-induced allergy (27) and pollen-associated food allergy (40). The aim of priming with IL-3 is to increase sensitivity of the assay by enhancing the CD63 up-regulation by allergen (22). This would be of interest when the allergenic stimulus is weak; however, IL-3 also increases nonspecific basophil activation, thus decreasing specificity (22). This might explain our findings of the two cases showing basophil activation while no symptoms of *Anisakis* allergy were present. These two samples showed a negative result when challenged with a lower concentration of crude extract. Furthermore, we have also found two samples with high basal activation and we consider that priming with IL-3 may be at the basis of this finding. Unfortunately, we were unable to reevaluate these cases and to perform the assay without IL-3. Conversely, a previous report has shown no CD63 up-regulation by IL-3 in purified basophils (41). Taking these data into account, we consider that more information about effects of IL-3 in basophil activation needs to be collected.

In conclusion, detection of *A. simplex*-induced basophil activation by flow cytometry is a useful laboratory technique for the diagnosis of anisakidosis. The technique provides additional information when clinical relevance of a positive specific IgE result is unclear.

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